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2017-05

Baumgartner , S , Mensink , R P , De Smet , E , Konings , M , Fuentes , S , de Vos , W M & Plat , J 2017 , ' Effects of plant stanol ester consumption on fasting plasma oxy(phyto) sterol concentrations as related to fecal microbiota characteristics ' , Journal of Steroid Biochemistry and Molecular Biology , vol. 169 , pp. 46-53 . https://doi.org/10.1016/j.jsbmb.2016.02.029

http://hdl.handle.net/10138/235588
https://doi.org/10.1016/j.jsbmb.2016.02.029

unspecified
publishedVersion

Journal of Steroid Biochemistry & Molecular Biology 169 (2017) 46–53

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

Effects of plant stanol ester consumption on fasting plasma oxy(phyto) sterol concentrations as related to fecal microbiota characteristics

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ARTICLE INFO

Article history:
Received 22 January 2016
Received in revised form 18 February 2016
Accepted 26 February 2016
Available online 3 March 2016

Keywords:
Plant stanols
Oxyphytosterols
Oxycholesterols
Gut microbiota

ABSTRACT

Information regarding dietary effects on plasma oxyphytosterol concentrations as well as on the origin of oxyphytosterols is scarce. We hypothesized that plant sterols are oxidized in the intestinal lumen, mediated by microbial activity, followed by uptake into the circulation. To address this hypothesis, we carried out, a randomized, double blind, crossover study in 13 healthy subjects, who consumed for 3 weeks control and plant stanol ester enriched margarines (3.0 g/d plant stanols) separated by a 4-week wash-out period. Plasma oxy(phyto)sterols were determined via GC–MS/MS, while microbiota analyses were performed on fecal DNA using a phylogenetic microarray to assess microbial composition and diversity. Plasma plant sterol concentrations did not correlate with plasma oxyphytosterols concentrations at baseline. Plant stanol consumption reduced serum sitosterol and campesterol concentrations (-37% and -38%), respectively ($p < 0.001$), as well as plasma concentrations of 7β-OH-campesterol (-24%; $p < 0.05$), 7β-OH-sitosterol (-17%; $p < 0.05$) and 7-keto-sitosterol (-13%; $p < 0.05$). Although the intestinal microbiota composition and diversity of the faecal contents were not different between the two periods, we observed significant correlations between several specific bacterial groups and plasma plant sterol, but not with plasma oxyphytosterol concentrations. In conclusion, plant stanol ester consumption reduced serum plant sterol and plasma oxyphytosterol concentrations, while intestinal microbiota composition and diversity were not changed. To definitely answer the effects of microbiota on oxyphytosterol formation, future studies could examine oxyphytosterol concentrations after changing intestinal microbial composition or by measuring intestinal oxyphytosterol formation after providing labelled non-oxidized plant sterols.

Contents

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http://dx.doi.org/10.1016/j.jsbmb.2016.02.029
0960-0760/

1. Introduction

Cholesterol and plant sterols can be oxidized, which results in the formation of a variety of different oxycholesterol and oxyphytosterol metabolites. While oxycholesterol metabolism and formation has been reviewed extensively [1–3], less is known about oxyphytosterols in this respect. For example, not much information is available on the effects of diet on plasma oxyphytosterol concentrations as well as on the site of oxidation of plant sterols. Husche et al. reported a significant 87% increase in fasting 7β-OH-sitosterol concentrations in healthy subjects after consumption of 3.0 g/d plant sterol enriched margarine for 4 weeks [4]. In contrast, we were not able to show changes in fasting oxyphytosterol concentrations after plant sterol consumption for 4 weeks despite increased fasting plant sterol concentrations. However, 7β-OH-campesterol concentrations and – when standardized for cholesterol – also 7-keto-campesterol concentrations were decreased after plant stanol consumption [5].

Even though plasma oxyphytosterol concentrations have been determined in several studies [4–7], the origin of circulating oxyphytosterols is unclear. Theoretically, plasma oxyphytosterol concentrations can result from absorption of oxyphytosterols as present in the diet, from in vivo oxidation of non-oxidized plant sterols within the circulation and tissues or alternatively, from oxidation of non-oxidized plant sterols that are present in the enterocytes or in the intestinal lumen. If circulating oxyphytosterols origin from intestinal oxidation, changes in microbiota composition could affect oxyphytosterol formation and consequently plasma oxyphytosterol concentrations. This can also explain the reduction in oxyphytosterol concentrations after plant stanol consumption, since plant stanol esters not only inhibit absorption of non-oxidized plant sterols [8] but possibly also that of oxidized plant sterols [5]. Also our finding that type II diabetics have higher oxyphytosterol concentrations as compared to controls (unpublished data), combined with the knowledge that type II diabetics are characterized by modified microbiota composition [9] suggests that microbiota might play a role in oxyphytosterol formation. The intestinal microbiota can regulate host metabolism at multiple levels and recent studies ascribe a potential role to the gut microbiota in the development of metabolic diseases such as obesity, insulin resistance and cardiovascular diseases (CVD) [10–12]. However, not much is known on the involvement of intestinal microbiota in cholesterol metabolism and whether plant sterol or plant stanol consumption indeed has an effect on the microbiota. In one publication from 1999, it was described that the daily consumption of a plant sterol enriched margarine did not affect the metabolic activities of the gut microflora [13]. This research was carried out as part of a safety evaluation of habitual plant sterol use. Since that time, technologies to assess intestinal microbiota composition and their effects on host metabolism have improved drastically. Therefore, we examined in this randomized placebo-controlled trial 1) effects of plant stanol consumption on plasma oxyphytosterol and oxycholesterol concentrations, 2) effects of plant stanol consumption on the intestinal microbiota composition and diversity, and 3) associations between circulating plasma plant sterol/stanol concentrations, plasma oxy(phyto)sterol concentrations and microbiota characteristics.

2. Subjects and methods

2.1. Subjects, diets and design

Details of the study design and the study population have been described previously [14,15]. Briefly, subjects were recruited in Maastricht and surroundings and met the following criteria:

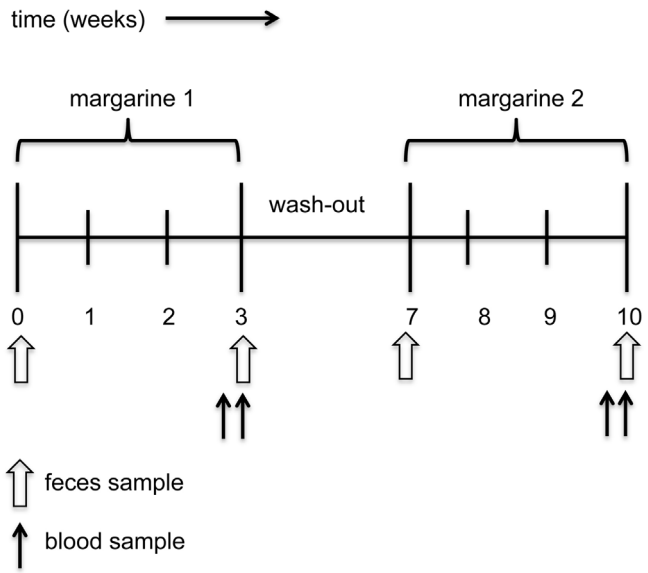

Fig. 1. Outline of study design.

18–60 years of age, body mass index between 20 and 30 kg/m^2, no history of diabetes, cardiovascular or gastrointestinal disorders and no use of lipid-lowering medication or a (medically) prescribed diet. In addition, mean serum total cholesterol concentrations were <7.8 mmol/L, as determined during two screenings visits. All subjects gave written informed consent before entering the study. The Medical Ethical Committee of the Maastricht University Medical Center (MUMC) approved the protocol and the trial is registered at clinicaltrials.gov as NCT01574417.

Subjects participated in a randomized placebo-controlled crossover trial, which consisted of two intervention periods of 3 weeks, separated by a 4-week washout period (Fig. 1) and were stratified for age, gender and BMI. Participants were randomly allocated to start with the control or plant stanol intervention. Subjects were asked to consume margarine enriched with plant stanol esters (3 g/day) or control margarine for 3 weeks. For this, subjects were asked to replace their own spread with the test margarine (70% fat) of which 20 g had to be consumed on a daily basis, which provided no, or 3.0 g of plant stanols per day. The margarines were packed in tubs of 140 g each, providing margarine for 7 days. The margarines were similar in color and taste and the tubs were color coded to blind both subjects and investigators. All volunteers received instructions to consume the margarines divided over two meals, i.e. at lunch and diner. The margarines were all rapeseed oil based with saturated soy sterols as source of the plant stanols, which were provided as fatty acid esters made by trans esterification of free plant stanols with rapeseed oil fatty acids. Plant stanol mixtures were obtained by saturation of sitosterol, campesterol and stigmasterol, resulting in sitostanol ester (76%) and campestanol ester (22%) (Raisio group, Raisio, Finland).

Subjects visited the university at the start of each 3-week intervention period and twice at the end of each period. Feces were collected at the start and end of each intervention period and blood was sampled on 2 occasions at the end of each period separated by 3 days. After each intervention period, subjects completed a validated food frequency questionnaire (FFQ) [16], which was checked by a certified dietician to calculate energy and nutrient intakes using the Dutch food composition Table Subjects were asked not to change their dietary habits, physical activity level and alcohol intake during the study.

2.2. Blood sampling and feces collection

Blood was sampled in a clotting tube and an EDTA tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA). Serum was obtained from the clotting tube by low-speed centrifugation at 1300*g* for 15 min at room temperature, at least half an hour after venipuncture and was used for the analysis of lipid, (apo) lipoprotein and plant sterol, stanol and cholesterol precursor concentrations. EDTA plasma was obtained by low-speed centrifugation at 1300*g* for 15 min at 4 °C and was used for the analysis of oxysterol and oxyphytosterol concentrations. All serum and plasma samples were snap frozen in liquid nitrogen and stored at −80 °C until analysis. All samples from one subject were analyzed with the same analytical run. Fecal samples were collected in containers and were stored at −80 °C until being analyzed.

2.3. Analyses

2.3.1. Lipid and lipoproteins

Total cholesterol (TCH) (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany), high-density lipoprotein cholesterol (HDL-C) (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany) after precipitation of apoB-containing lipoproteins with phosphotungstic acid and magnesium ions, and TAG concentrations, with correction for free glycerol, were analysed in serum enzymatically (GPO-Trinder; Sigma-Aldrich Corp., St. Louis, MO, USA). Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated according to the Friedewald equation [17]. Apolipoprotein concentrations (apoA-1 and apoB100) were analysed using highly sensitive immunoturbidimetric assays (Horiba ABX, Montpellier, France). Lipid and lipoprotein concentrations were measured in the two samples obtained at the end of each period and averaged for data analysis.

2.3.2. Plant sterols, plant stanols, non-cholesterol sterols and oxy (phyto)sterols

Plant sterol (sitosterol, campesterol), plant stanol (sitostanol, campestanol), cholesterol precursors (lathosterol, desmosterol) and cholesterol absorption marker (cholestanol) were analyzed by gas-liquid chromatography-mass spectrometry (GC–MS) as described previously [18]. Non-cholesterol sterol concentrations are expressed as mg/dL. Oxysterols (7α-Hydroxy(OH)-cholesterol, 7β-OH-cholesterol and 7-keto-cholesterol) and oxyphytosterols (7α-OH-campesterol, 7α-OH-sitosterol, 7β-OH-campesterol, 7β-OH-sitosterol, 7-keto-campesterol and 7-keto-sitosterol) were analyzed by gas chromatography-mass spectrometry (GC–MS-MS) according a triplequad procedure as before [19]. During work-up procedures, 5 μl BHT (0.25 mg/mL) per sample was added to avoid autoxidation. Plant sterol, plant stanol, non-cholesterol sterol and oxy(phyto) sterol concentrations were measured in the samples obtained at the end of each intervention periods.

2.3.3. Fecal microbiota

Total DNA from fecal samples was isolated using the repeated bead beating method as described previously [20]. The Human Intestinal Tract chip (HITChip), a phylogenetic microarray containing probes for over 1100 known intestinal phylotypes was used for the semi-quantitative characterization of the fecal microbiota [21]. Processing of microarray data was performed using a set of R-based scripts (http://r-project.org) in combination with a custom designed database under the MySQL database management system (http://www.mysql.com) as previously described [22]. Bacterial diversity (Shannon index of diversity), including richness and evenness, was calculated based on the total oligonucleotide signals.

2.4. Statistics

Endpoint concentrations were compared with a paired *t*-test to evaluate the effect of plant stanol ester consumption on lipid, lipoprotein, plant sterol/stanol and oxy(phyto)sterol concentrations. Endpoint values of microbiota diversity were also evaluated by a paired *t*-test and differences between the 130 genus-like groups included in the HITChip were assessed by Mann-Whitney *U* test (Wilcoxon for paired data). Differences at baseline in the control period in microbiota diversity between males and females and two BMI categories (≤25 and >25 kg/m^2) were evaluated by Student's *t*-test and differences in microbiota composition were assessed as described above.

Associations between endpoint concentrations from the control and plant stanol ester periods in individual plasma oxyphytosterol concentrations were determined by Pearson product-moment correlation coefficient. Cross-sectional associations in the control period between microbiota composition and different sterol/stanol and oxy(phyto)sterol concentrations and subject characteristics (BMI and age) were performed by Spearman's rank correlation coefficient as implemented in R (version 3.2.0). Correction for multiple testing was performed by the Benjamin-Hochberg

method with false discovery rate (FDR) at 5% [23]. All data are presented as means ± standard deviations (SD). Results were considered to be statistically significant if $p < 0.05$ and all statistical analyses were performed using SPSS 20.0 for Mac Os X or higher (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Baseline characteristics

Of the initial 18 subjects that completed the study, samples of 13 subjects were available for the analyses described here. Their baseline characteristics are shown in Table 1. Dietary intakes of macro- and micronutrients were comparable in both intervention periods and body weights remained stable throughout the study (data not shown).

3.2. Serum lipid, lipoprotein and non-cholesterol sterol concentrations

Serum lipid and lipoprotein concentrations are shown in Table 2. Compared with the control period, serum total cholesterol concentrations decreased by 7.0% ($p < 0.001$) and serum LDL-C concentrations decreased by 9.7% ($p < 0.001$) after three weeks plant stanol ester consumption. In line with the effects on serum LDL cholesterol, serum apolipoprotein B100 concentrations were decreased by 8.7% ($p < 0.01$), while no significant differences were found in serum TAG, HDL-C, and apolipoprotein A1 concentrations after plant stanol consumption as compared with the control period. After plant stanol consumption, serum sitosterol concentrations decreased by 0.12 ± 0.05 mg/dL (-37%; $p < 0.01$) and campesterol concentrations decreased by 0.20 ± 0.06 mg/dL (-38%; $p < 0.01$) as compared with the control period. In addition, serum plant stanol concentrations increased after plant stanol intake, i.e. sitostanol concentrations increased by 33.9. ± 9.06 μg/dL (300%; $p < 0.01$) and campestanol concentrations increased by 13.8 ± 4.8 μg/dL (194%; $p < 0.01$). Lathosterol and desmosterol concentrations (surrogate markers of endogenous cholesterol synthesis) and the cholestanol concentration (marker of cholesterol absorption) (cholestanol) were not significantly different after 3-week plant stanol ester consumption (Table 2).

3.3. Plasma oxyphytosterol and oxycholesterol concentrations

Table 3 shows plasma oxyphytosterol and oxycholesterol concentrations after 3 weeks consumption of control and plant stanol ester enriched margarine. 7α-OH-sitosterol and 7α-OH-campesterol concentrations were comparable after both intervention periods. Plant stanol consumption reduced 7β-OH-sitosterol concentrations by 0.18 ± 0.26 (-17%; $p < 0.05$) and 7β-OH-campesterol concentrations by 0.13 ± 0.15 (-24%; $p < 0.05$). Concentrations of 7keto-sitosterol were reduced by 1.41 ± 1.82 (-13%; $p < 0.05$), while 7keto-campesterol concentrations were not significantly reduced after plant stanol consumption compared with the control period. The sum of the analyzed serum oxyphytosterol concentrations was significantly lowered by 15% ($p < 0.001$). Except for reduced 7α-OH-cholesterol concentrations (-17%; $p < 0.01$), plant stanol consumption did not change concentrations of the other oxycholesterol isoforms as compared with the control period. Serum non-oxidized plant sterol concentrations and plasma oxyphytosterol concentrations did not correlate at baseline (after control intervention) or after plant stanol consumption. Changes after plant stanol consumption in individual plasma oxyphytosterol concentrations were significantly correlated or tended to correlate with each other (Supplemental Table 1).

Table 1
Baseline characteristics of the subjects.

Age (years)	30.3 ± 11.6
Male/Female (n)	6/7
BMI (kg/m^2)	23.5 ± 2.5
Glucose (mmol/L)	5.2 ± 0.4
Total cholesterol (mmol/L)	5.1 ± 1.2
LDL cholesterol (mmol/L)	3.1 ± 0.9
HDL cholesterol (mmol/L)	1.5 ± 0.4
Triacylglycerol (mmol/L)	1.1 ± 0.4

Values are means ± SD (N = 13).

3.4. Fecal microbiota

Fig. 2A presents the relative abundances of microbial phyla at baseline of the whole group as well as subdivided for gender or for two BMI categories representing normal weight and overweight (i.e. ≤25 and >25 kg/m^2 respectively). The largest phyla are represented by the Firmicutes, followed by Bacteroidetes, Actinobacteria and Proteobacteria. The phylum Firmicutes was subdivided into *Clostridium* clusters. Males and females had a comparable fecal microbiota composition, except for the bacterial groups related to *Clostridium nexile* and *Klebisiella pneumonia*, which were more abundantly present in females ($p < 0.05$). The two BMI categories differed regarding 5 bacterial groups (related to *Atopobium, Bulleidia moorei, Clostridium (sensu stricto), Uncultured Mollicutes and Weissella.*; $p < 0.05$), which were more abundantly present in the lower BMI category (≤25 kg/m^2). However, application of the FDR correction for multiple testing suggested that the differences observed could also be chance findings. Fig. 2B represents the relative abundances of microbial phyla at the end of the control and plant stanol ester period. There was no change in fecal microbiota composition after three weeks consumption of plant stanol compared with the control period in this population of healthy volunteers. In addition, there were no effects of plant stanol intake in the composition of the fecal microbiota when the analyses were repeated for the two BMI or gender groups separately (data not shown).

Fig. 3A shows the fecal microbiota diversity at baseline (presented by the Shannon index) in all subjects and separated for gender and two BMI categories (≤ 25 and >25 kg/m^2). Diversity was comparable between males and females, and between the two BMI categories. Fecal microbiota diversity did not change after consumption of a plant stanol enriched margarine for 3 weeks compared with the control group (Fig. 3B).

Associations between the 130 genus-like groups identified in the HITChip microarray and serum plant sterol/stanol, oxy(phyto) sterol concentrations and subject characteristics (BMI and age) generally resulted in high FDR values. Therefore, only significant correlations ($p < 0.05$ and FDR < 0.3) are reported in Table 4. In general, serum plant sterol concentrations correlated positively with a variety of investigated bacterial groups, belonging to the clostridia (phylum Firmicutes). In addition, negative correlations were found between serum plant sterol concentrations and *Clostridium colinum* and related species. None of the plasma oxysterol or oxyphytosterol concentrations correlated with any bacterial groups. For serum plant stanol concentrations, only serum sitostanol correlated positively with bacterial groups belonging to the phylum Bacteroidetes and serum campestanol concentrations correlated negatively with *Eubacterium ventriosum* and related species. There were positive correlations identified between cholestanol, a surrogate marker for cholesterol absorption, and several bacterial groups, including those related to *Clostridium cellulosi, Papillibacter cinnamivorans,* Outgrouping *Clostridium* cluster XIVa and *E. ventriosum*. Desmosterol

Table 2
Serum lipid, lipoprotein, plant sterol, plant stanol and non-cholesterol sterol concentrations after consumption of control and plant stanol ester enriched margarines.

	Control	Plant stanol	Change (Stanol—Control)
Total cholesterol (mmol/L)	5.36 ± 0.94	4.98 ± 0.91	−0.46 ± 0.41[b]
LDL cholesterol (mmol/L)	3.17 ± 0.89	2.83 ± 0.75	−0.39 ± 0.35[b]
HDL cholesterol (mmol/L)	1.57 ± 0.32	1.60 ± 0.36	0.03 ± 0.14
Triacylglycerol (mmol/L)	1.32 ± 0.41	1.21 ± 0.48	−0.12 ± 0.26
ApoB100 (g/L)	0.93 ± 0.23	0.85 ± 0.21	−0.09 ± 0.06[a]
ApoA1 (g/L)	1.48 ± 0.24	1.47 ± 0.24	−0.01 ± 0.11
Sitosterol (mg/dL)	0.32 ± 0.09	0.21 ± 0.07	−0.12 ± 0.05[a]
Campesterol (mg/dL)	0.52 ± 0.14	0.33 ± 0.12	−0.20 ± 0.06[a]
Sitostanol (ug/dL)	11.67 ± 1.78	46.29 ± 9.70	33.90 ± 9.06[a]
Campestanol (ug/dL)	7.43 ± 1.53	21.62 ± 5.42	13.75 ± 4.82[a]
Lathosterol (mg/dL)	0.30 ± 0.13	0.30 ± 0.12	0.00 ± 0.08
Cholestanol (mg/dL)	0.51 ± 0.05	0.49 ± 0.06	−0.02 ± 0.03
Desmosterol (mg/dL)	0.13 ± 0.04	0.13 ± 0.05	0.00 ± 0.01

Values are means ± SD (N = 13).
[a] $p < 0.001$.
[b] $p < 0.01$.

Table 3
Oxy(phyto)sterol concentrations after consumption of control and plant stanol ester enriched margarines.

	Control	Stanol	Change (Stanol—Control)
7α-OH-sitosterol (ng/mL)	0.42 ± 0.21	0.33 ± 0.12	−0.11 ± 0.25
7α-OH-campesterol (ng/mL)	0.36 ± 0.18	0.24 ± 0.08	−0.13 ± 0.22
7β-OH-sitosterol (ng/mL)	0.77 ± 0.28	0.59 ± 0.15	−0.18 ± 0.26[b]
7β-OH-campesterol (ng/mL)	0.46 ± 0.16	0.33 ± 0.10	−0.13 ± 0.15[b]
7keto-sitosterol (ng/mL)	8.82 ± 2.70	7.48 ± 1.77	−1.41 ± 1.82[b]
7keto-campesterol (ng/mL)	1.23 ± 0.39	1.00 ± 0.35	−0.23 ± 0.52
Sum oxyphytosterols (ng/mL)	12.02 ± 3.39	9.95 ± 2.29	−2.18 ± 2.77[a]
7α-OH-cholesterol (ng/mL)	64.0 ± 26.4	53.0 ± 22.6	−12.2 ± 14.3[b]
7β-OH-cholesterol (ng/mL)	20.6 ± 6.0	18.8 ± 3.5	−2.0 ± 5.5
7keto-cholesterol (ng/mL)	22.0 ± 9.21	19.6 ± 2.5	−2.9 ± 8.7
24-OH-cholesterol (ng/mL)	31.4 ± 20.2	27.4 ± 7.9	−4.3 ± 16.7
27-OH-cholesterol (ng/mL)	45.0 ± 17.3	42.6 ± 16.4	−2.5 ± 7.4

Values are means ± SD (N = 13).
[a] $p < 0.001$.
[b] $p < 0.05$.

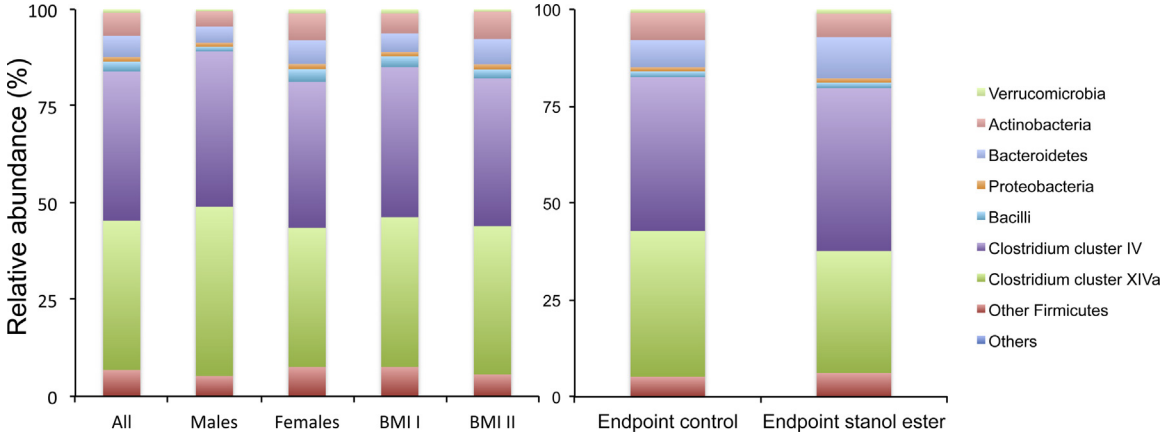

Fig. 2. No difference in relative abundances of microbial phyla at baseline and after consumption of control or plant stanol ester enriched margarine.
Data is presented for all subjects and for gender and BMI category (BMI I: ≤25 and BMI II: >25 kg/m^2) at baseline (left) and at the end of both intervention periods (right).

(cholesterol precursor) correlated positively with bacteria related to *Streptococcus mitis*, but negatively with Uncultured *Mollicutes* and *Akkermansia*. However, no correlations were found with serum lathosterol (surrogate marker for cholesterol synthesis). Finally, some bacterial groups correlated positively with age, indicating that these are more abundantly present with higher age, while BMI correlated negatively with Uncultured *Chroococcales* and Uncultured *Mollicutes*. Indeed, the latter bacterial group was also less abundantly present in the higher BMI category ($p < 0.05$, without FDR correction).

4. Discussion

Absorption rates for plant stanols from the intestinal lumen into the circulation are very low (<0.5%) and due to rapid clearance from the circulation by the liver and secretion into bile via hepatic

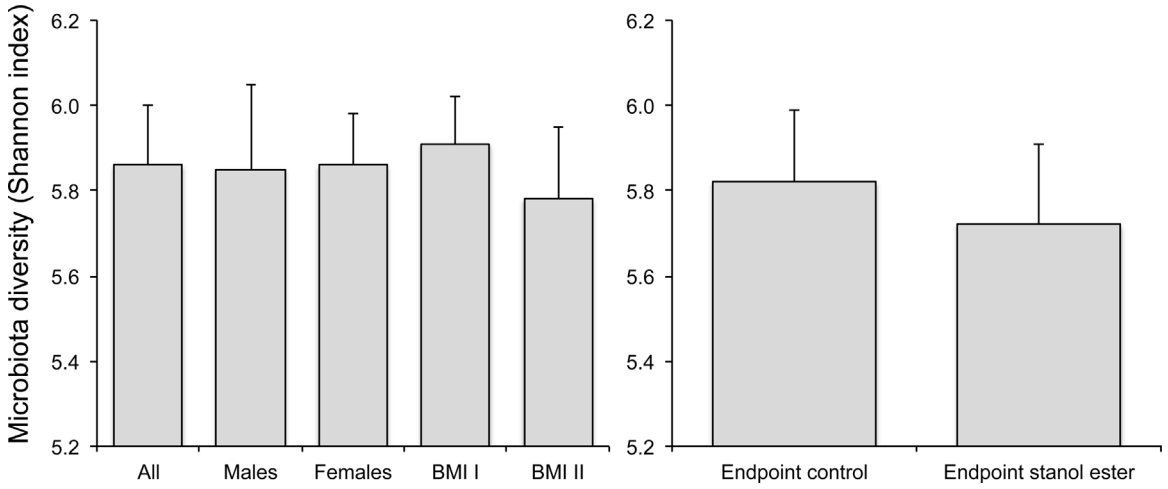

Fig. 3. No difference in microbiota diversity at baseline in all subjects and after consumption of control or plant stanol ester enriched margarine.
Data is presented for all subjects and for gender and BMI category (BMI I: ≤25 and BMI II: >25 kg/m^2) at baseline (left) and at the end of both intervention periods (right).

Table 4
Associations of bacterial groups at a genus-like level with serum plant sterol/stanol concentrations, BMI and age.

Phylum/Class	Genus-like level	Variable	Correlation coefficient	p-value
Actinobacteria				
	Collinsella	Desmosterol	0.483	0.007
Bacteroidetes				
	Bacteroides splachnicus et rel.	Sitostanol	0.507	0.004
	Prevotella oralis et rel.	Sitostanol	0.559	0.001
	Tannerella et rel.	Sitostanol	0.534	0.002
	Uncultured Bacteroidetes	Sitostanol	0.568	0.001
Cyanobacteria				
	Uncultured Chroococcales	BMI	−0.483	0.007
Firmicutes				
Bacilli	*Streptococcus mitis et rel.*	Desmosterol	0.523	0.003
C. cluster IV	*Clostridium cellulosi et rel.*	Sitosterol	0.699	0.000
	Clostridium cellulosi et rel.	Campesterol	0.694	0.000
	Clostridium cellulosi et rel.	Cholestanol	0.496	0.005
	Clostridium leptum et rel.	Sitosterol	0.545	0.002
	Papillibacter cinnamivorans et rel.	Age	0.509	0.004
	Papillibacter cinnamivorans et rel.	Cholestanol	0.486	0.006
	Ruminococcus bromii et rel.	Campesterol	0.624	0.000
	Ruminococcus bromii et rel.	Sitosterol	0.519	0.003
C. cluster XI	*Anaerovorax odorimutans et rel.*	Sitosterol	0.507	0.004
	Clostridium difficile et rel.	Sitosterol	0.529	0.003
C. cluster XIVa	*Clostridium colinum et rel.*	Sitosterol	−0.550	0.002
	Clostridium colinum et rel.	Campesterol	−0.519	0.003
	Clostridium nexile et rel.	Desmosterol	0.488	0.006
	Coprococcus eutactus et rel.	Campesterol	0.491	0.006
	Dorea formicigenerans et rel.	Campesterol	0.487	0.006
	Eubacterium ventriosum et rel.	Campestanol	−0.506	0.004
	Outgrouping clostridium cluster XIVa	Cholestanol	0.483	0.007
	Ruminococcus lactaris et rel.	Campesterol	0.620	0.000
	Ruminococcus lactaris et rel.	Age	0.516	0.004
C. cluster XVI	*Eubacterium biforme et rel.*	Cholestanol	0.549	0.002
Unc. Mollicutes	*Uncultured Mollicutes*	Desmosterol	−0.529	0.003
	Uncultured Mollicutes	BMI	−0.527	0.003
Verrucomicrobia				
	Akkermansia	Desmosterol	−0.742	0.000

Grey shading represent negative correlations (N = 13).

ABCG5/G8 transporters, plasma plant stanol concentrations are low and range from 0.002 to 0.012 mg/dL [24,25]. The presence of plant stanols in the intestinal lumen interferes with intestinal absorption rates of cholesterol and of plant sterols, which reduces serum cholesterol and plant sterol concentrations. In the present study, we observed a reduction of 9.7% in serum LDL-C concentrations at a daily intake of 3 g stanol/day, which is in agreement with a recent meta-analysis estimating an effect of 10.4% (CI: −11.7%; −9.1%) in 11 studies after an average intake of 2.6 g/d of plant stanols [26]. In addition, serum sitosterol and campesterol concentrations were reduced by ~38%, which is comparable with observed reductions in previous studies [27]. While the lowering effect of plant stanols on cholesterol and plant sterol concentrations has been observed in many studies, their effect on the oxidation products of cholesterol and plant sterols, i.e. oxycholesterols and oxyphytosterols have been less extensively studied. Previously, we demonstrated a decrease of 0.07 ± 0.17 ng/mL (13.6%) in 7β-OH-campesterol in 43 healthy

subjects after consuming a plant stanol-enriched margarine for 4 weeks (3 g/d) [5]. In the current study, we found a stronger decrease in 7β-OH-campesterol concentrations, i.e. a reduction of -0.13 ± 0.15 (-24%). In addition, other ROS-generated oxyphytosterols (7β-OH-sitosterol and 7keto-sitosterol) were also reduced by plant stanol consumption for 3 weeks and the sum of oxyphytosterols was decreased with ~15%. The same food product was used in both studies, namely a plant stanol enriched margarine, providing 3 g of plant stanols per day. In addition, both studies were performed in a healthy population with a comparable age range (18–69 years) and lipid profile (TC <6.1 mmol/L). Moreover, the present study evaluated the effect of plant stanols after 3 weeks, while the previous study had an intervention period of 4 weeks, but it is unlikely that this difference in duration can explain why plasma oxyphytosterol concentrations decreased to a greater extent.

Plant sterol and oxyphytosterol concentrations did not correlate at baseline and reductions in serum plant sterol concentrations were also not correlated with changes in plasma oxyphytosterol concentrations after plant stanol consumption. This is in accordance with previous data, showing that increases in serum plant sterol concentrations and plasma oxyphytosterol concentrations also do not correlate. Overall, these data show that neither an increase nor a decrease in non-oxidized plant sterol concentrations influences circulating oxidized plant sterols.

Therefore the question remains where the circulating oxyphytosterols come from? Hypothetically, circulating oxyphytosterol concentrations can originate from 1) absorption of oxyphytosterols that are already present in the diet, from 2) oxidation of non-oxidized plant sterols within the circulation and/or tissues or from 3) oxidation in the enterocytes and/or intestinal lumen followed by uptake into the circulation. Previously, we have postulated that plasma oxyphytosterol concentrations might not be related to absorption of oxyphytosterols present in the diet, since a higher dietary intake of oxyphytosterols (0.7 mg vs. 0.1 mg per day) did not result in higher plasma concentrations after 4 weeks. Furthermore, we were able to show differences in oxyphytosterol concentrations between subjects with low and high oxidative stress [28] and between healthy subjects and type 2 diabetic patients (unpublished data). It is unlikely that these different oxyphytosterol concentrations are attributed to differences in oxyphytosterol absorption rates. Oxidation of plant sterols in the circulation and/or tissues might also be a source of plasma oxyphytosterol concentrations, but in general changes in plasma plant sterol concentrations after plant sterol or plant stanol consumption do not correlate with changes in plasma oxyphytosterol concentrations [4]. In addition, apoE$^{-/-}$ mice that received daily intraperitoneal injections over 4 weeks with non-oxidized cholesterol or sitosterol did not show an increase in plasma oxycholesterol or oxyphytosterol concentrations [29], implying that substrate oxidation in the circulation might also not be the most important source of plasma oxyphytosterol concentrations. As a final possibility we postulated that circulating oxyphytosterols might origin from oxidation of plant sterols within the enterocyte or in the intestinal lumen. In this context the microbiota might play a crucial role in the formation of oxyphytosterols. For this reason, an objective of this study was to assess whether a plant stanol-induced decrease in plasma oxyphytosterol concentrations might be attributed to changes in intestinal microbiota composition. However, despite reduced plasma oxyphytosterol concentrations, 3 weeks of plant stanol consumption did not affect microbiota composition or diversity. Furthermore, fecal microbiota composition and diversity were comparable between men and women and between different BMI categories, and plant stanol consumption did not induce any changes in these subgroups. In addition, there were no cross-sectional correlations between plasma oxyphytosterol concentrations and bacterial groups at genus-like level. Since microbiota composition and diversity was not changed after plant stanol ester consumption, we cannot draw any firm conclusions related to the question whether circulating oxyphytosterol concentrations might origin from intestinal oxidation. For this reason, it would be highly interesting for future research to assess whether a drastic change in microbiota composition, for example by an antibiotic therapy, affects plasma oxyphytosterol concentrations. In addition, a study where labelled non-oxidized plant sterols are provided and oxyphytosterol concentrations are measured in luminal content will also give more information whether oxyphytosterol formation takes place in the intestinal lumen.

While oxidized plant sterols did not correlate with intestinal microbiota, there were several cross-sectional correlations between serum plant stanol/sterol concentrations and bacterial groups. Serum sitostanol correlated positively with bacterial groups belonging to the phylum Bacteroidetes, while serum campestanol concentrations correlated negatively with bacteria related to *E. ventriosum* (phylum Firmicutes). *E.ventriosum* may produce butyrate, but its abundance has been reported to be higher in Japanese obese subjects [30] and correlated to polyunsaturated fatty acids-derived metabolites in Belgian women with metabolic syndrome [31]. An increase in butyrate-producing bacteria may affect host energy metabolism by promoting energy harvest [32]. On the other hand, desmosterol (a cholesterol precursor) correlated negatively with *Akkermansia* (phylum Verrucomicrobia), a bacterium that has been linked to the prevention of obesity [33]. Although data is inconsistent, a relative higher abundance of Firmicutes has been associated with obesity and these associations might indicate potential connections between microbiota composition and markers of cholesterol metabolism. However, these are cross-sectional correlations and further evaluation of a potential involvement of microbiota in cholesterol metabolism would be interesting to explore into more detail in future research. To our knowledge, this is the first time that plasma plant stanol/sterol concentrations have been linked to microbiota composition and further research is warranted in this field.

Besides oxidized plant sterols, we also determined oxidized cholesterol concentrations, namely 7β-OH-cholesterol and 7keto-cholesterol concentrations (ROS-generated oxycholesterols) and 24-OH-cholesterol and 27-OH-cholesterol concentrations (enzymatically-generated oxycholesterols), while 7α-OH-cholesterol can be produced enzymatically as well as non-enzymatically. Both 24-OH-cholesterol and 27-OH-cholesterol are produced as an alternative mechanism to secrete cholesterol from brain and human plaques, respectively [34,35]. Therefore, concentrations of these oxycholesterols reflect a specific role for these products at a certain site and any change in cholesterol availability or ROS level does not influence their circulating concentrations. It is therefore not surprising that a change in serum TC concentrations did not affect 24-OH and 27-OH-cholesterol concentrations. In addition, also ROS-generated oxycholesterol concentrations were not changed after plant stanol consumption and changes in cholesterol and ROS-oxycholesterol concentrations after plant stanol consumption were also not correlated. This is in contrast to plasma oxyphytosterol concentrations, where 7β-OH- and 7keto-phytosterols were reduced after plant stanol consumption. Cholesterol is present in plasma lipoproteins and in cell membranes. Although cholesterol can be oxidized, its susceptibility for oxidation is much lower than polyunsaturated fatty acids that are also present in lipoproteins and cell membranes [1]. This notion, together with a modest decrease in serum TC concentrations compared to the large amount of cholesterol present in the body, most likely explains why ROS-generated oxycholesterol were not changed after plant stanol consumption for 3 weeks.

5. Conclusion

In conclusion, this study shows that consumption of plant stanol esters for 3 weeks reduced plasma oxyphytosterol concentrations without changing intestinal microbiota composition and diversity. Further research is needed to provide more insight whether circulating oxyphytosterol concentrations might be derived from neutral sterol oxidation in the gastro-intestinal tract. In this respect, it would be interesting to assess oxyphytosterol concentrations after changing microbial composition or to provide labelled non-oxidized plant sterols and to measure oxyphytosterol concentrations in luminal content.

Acknowledgements

The authors would like to thank all study participants for their cooperation and the technical and dietary staff for their support. The study was financially supported by Raisio, Finland. Part of this work was supported by the Spinoza Award and the SIAM Gravity Grant (024.002.002) of the Netherlands Organization of Scientific Research to WMdV.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2016.02.029.

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